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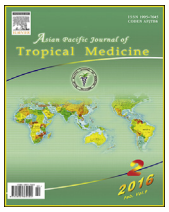

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Immunogenicity and immunizing protection effect of *GAMA* gene DNA vaccine on *Plasmodium berghei*

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ABSTRACT

Objective: To explore the effect of immunogenicity and immunizing protection of *GAMA* gene DNA vaccine, which was related with merozoite, ookinete and sporozoite invasion.

Methods: Gene fragments were obtained using PCR technique and eukaryotic expression vector (containing immunostimulatory sequence) was built. BALB/c mice were divided into PBS control group, empty vector control group and study group and were immunized at week 0, 3 and 6 respectively. Blood was collected 2 weeks after each immunization and serum was separated to detect the IgG, IgG1 and IgG2a levels. Spleen of mice was obtained for preparation of splenic mononuclear cell and the cytokine IL-4 and IFN-γ levels were detected. Indirect immunofluorescence and western blot were employed to verify the specificity of antiserum. Sporozoite and merozoite invasion were used respectively to detect the immune protective effect 2 weeks after the third immunization. Ookinete conversion rate *in vitro* and oocyst numbers of mosquito stomach were observed to evaluate the transmission-blocking levels.

Results: In GAMA DNA vaccine group: antiserum could be combined with recombinant protein specifically and green fluorescence signals of merozoite, ookinete and sporozoite were observable, while specific fragments and fluorescence signals were not observable in empty vector group. Compared with control group, specific IgG in DNA vaccine immunity group significantly increased ($P < 0.01$), and IgG1 and IgG2a all increased ($P < 0.01$). IL-4, IFN-γ content in study group significantly increased, compared with control group ($P < 0.01$). GAMA DNA vaccine immunity could not obviously block the erythrocyte-stage infection (caused by sporozoite invasion); compared with control group, liver worm load was slightly reduced ($P < 0.05$), and antiserum ookinete numbers (cultured *in vitro*) had no significant difference with oocyst numbers of mosquito stomach in DNA vaccine group.

Conclusions: GAMA has good antigenicity, which could stimulate the body to produce specific immune responses; while DNA vaccine immunity could not play a good protective effect, the effect of which is only limited to the slight reduction of liver worm load, and has no obvious erythrocyte-stage protective effect and transmission-blocking effect. Therefore, trying other immunization strategies for further research on the value of GAMA (as multi-stage antigen vaccine and multi-stage combined vaccine components of the life-cycle of plasmodium) is necessary.

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1. Introduction

Malaria is caused by plasmodium infection and was transmitted by female anopheles. As is estimated by World Health Organization, there are about 198 million cases around the world in 2013, among which, about 584000 people die from plasmodium infection (mainly for children under the age of five) [1]. The drug resistance of plasmodium and mosquitoes inhibits the comprehensive control of parasites and disease vectors, which makes it difficult to effectively control malaria [2–4]. Although large quantities of promising preclinical studies have been carried out, there is still no an effective vaccine which could prevent infection or at least improve malaria clinical disease development. RTS,S, which could induce the immune responses of circumsporozoite protein, is a kind of pre-erythrocytic stage vaccine aiming to prevent from the primary infection of liver cells and may be the first malaria vaccine to enter into market after finishing clinical trials [5]. Although RTS,S could prevent partial malaria infection, especially for children, and then lower the mortality rate of malaria [6], developing more effective vaccine for further eliminating malaria is still necessary [7,8].

DNA vaccine could produce protective immune responses to various pathogens [9–11]. Among them, there have been four animal vaccine products with license, namely, equine West Nile virus vaccine and infectious hematopoietic necrosis virus vaccine of fish for infection prevention, vaccine for the treatment of dog cancer and growth hormone gene therapy for increasing survival rate of suckling pig. These examples show that DNA vaccine is effective in various species [11]. DNA vaccine of plasmodium in the pre-erythrocytic stage, erythrocytic stage, sexual stage and multi-stage could also induce specific immune responses and protective effect [12–18]. Therefore, DNA vaccine should be one of the most effective methods for malaria prevention and control.

Plasmodium has complex life-cycle, which could cause mammalian host infection and mosquito (transmission carrier) infection, which indicates antigen uniqueness and diversity in the infection and replication stage and limited the effect of stage-specific vaccine [19]. Therefore, multi-stage malaria vaccine could achieve higher effects. Multi-stage vaccine is not only multiple antigens expressed in multiple stages but also single antigen expressed in multiple stages. GAMA is a kind of multi-stage expression antigen involved in invasion [20–23]. GAMA is anchored at the top recombiner of merozoite, which is a kind of GPI-anchored erythrocyte-binding protein, has expression in ookinete and has important effects during liver phasic development. The GAMA knockout type oocyst and sporozoite formation quantities of *Plasmodium berghei* (*P. berghei*) are respectively 22.0% and 0.3% of the wild type and knockout type sporozoite could not cause vertebrate infection [20]. In addition, antibody induced by GAMA (expressed by acellular wheat germ system) has 38% of invasive inhibition ability and 20% of growth inhibitory activity *in vitro* [22].

In this paper, DNA vaccine immunization strategy is employed to discuss immunogenicity and preventive effect of GAMA, with *P. berghei* as model. The immune effects after its immunization and the multiple-stage immune preventive effects are evaluated.

2. Materials and methods

2.1. Ethical approval

This study was in accordance with the China Medical University laboratory animal ethics standards and laboratory animal welfare technical operation standard website (http://pwc.cnilas.org/plus/list.php?tid=1) requirements.

2.2. Bioinformatics analysis

GAMA gene sequence data and gene expression profile of *P. berghei* were obtained from PlasmoDB URL (http://PlasmoDB.org; code: PBANKA_070190). SMART (Simple Modular Architecture Research Tools) was used for functional domain prediction. Antigen peptide was predicted by online antigen prediction software (http://imed.med.ucm.es/Tools/antigenic.pl) and LASERGENE software. Homologous sequence comparison [*Plasmodium falciparum* (PF3D7_0828800), *Plasmodium knowlesi* (PKH_050210), *Plasmodium yoelii (P. yoelii)* (PY17X_0702200), *Plasmodium chabaudi* (PCHAS_093610) and *Plasmodium vivax* (PVX_088910)] was conducted by CLUSTALX2 to analyze the sequence similarity.

2.3. Mice, plasmodium and mosquito

Specific pathogen free BALB/c mice (5–6 weeks) were bought from Liaoning Changsheng Biotechnology Co., LTD. *Anopheles stephensi (A. stephensi)* was breeded in mosquito room. *P. berghei* was used in this study. Sporozoite, which was used for invasion, was collected from infected mosquito salivary glands and was placed in 1640 culture medium (contained mice serum) [24,25].

2.4. Plasmid construction and purification

Gene sequence (removed from signal peptide and transmembrane domain) was designed as DNA vaccine component [amino acid site (aa) 22-602]. Termination codon TAA, immunostimulation sequence CpG sequence motif and enzyme digestion site-*Bgl*II were included in reverse primers, and enzyme digestion site-*Bam*HI was included in forward primers. The obtained target fragments were connected to the eukaryotic expression vector VR1020. Fragments [amino acid site (aa) 46-552] were cloned into prokaryotic expression vector for recombinant protein-PbGAMA expression. Gene sequence was analyzed by the Vector NTI Advance11.5.1 to make sure the correct code-reading and the expression of fusion tags. DNA vaccine vector was extracted by Endotoxinfree Plasmid Maxi Kit (Tiangen) and was dissolved in PBS, and concentration of which was adjusted to 1 mg/mL.

2.5. Recombinant protein expression

Recombinant protein-PbGAMA was contained in *Escherichia coli* Rosetta-gamiB (DE3), which was expressed using 1.0 mM of isopropylthiogalactoside (20 °C) for 6 h. Recombinant protein was verified by western blot and was purified by ion affinity chromatography using histidine choosing cobalt resin (Sangon).

2.6. Inoculation

BALB/c mice were randomly divided into 3 groups (PBS control group, empty vector VR1020 control group and VR1020-PbGAMA immune group). Intramuscular injection of immune vaccine was carried out at quadriceps femoris site, with once every three weeks, total immunization for 3 times. Two weeks after each immunization, serum was collected from 3 mice in each group and spleen cells were separated for cultivation.

2.7. Analysis of western blot and indirect immunofluorescence

Western blot was analyzed using prokaryotic expressive recombinant protein to verify that DNA vaccine immunization has produced PbGAMA specific antibody. After 12% of SDS-PAG electrophoresis, recombinant protein was transferred onto the surface of PVDF membrane (Merck). The membrane was sealed by PBS (contained 5% of skim milk powder) and was nurtured in VR1020-PbGAMA immune serum and control serum (1:100 dilution) for 1 h respectively. After PBST rinsing, Goat Anti-Mouse IgG (H+L) (1:10 000 dilution, HRP labeled) (Jackson ImmunoResearch Labs) was added for nurturing. After rinsing, protein was fixed on the membrane and ECL enhanced chemiluminescence reagent kit (Thermo) was used for imaging analysis by imager (Tanon). Protein molecular weight relative size was compared and evaluated using PageRuler Prestained protein molecular weight standard (Thermo). PbGAMA polyclonal antiserum was used to carry out the indirect immunofluorescence of agamont, ookinete and sporozoite in *P. berghei* and to verify the ability of antiserum combining with parasite surface. Agamont, ookinete were cultivated in accordance with methods illustrated in literature [26] and sporozoite was collected from the infected mosquito salivary glands. Indirect immunofluorescence was performed in accordance with descriptions in literature [27].

2.8. Antibody analysis

Blood was collected from eyeballs two weeks after each immunization and serum was collected centrifugally for storing at −70 °C. Specific IgG and IgG1, IgG2a were detected by ELISA testing [27]. Brief speaking, recombinant protein-PbGAMA (10 μg/mL) was dissolved in PH9.6 coating solution, with 100 μL per well for coating ELISA plate. Coating ELISA plate was washed by PBST (PBS: containing 0.05% of Tween 20) and sealing solution (PBS: containing 1% of BSA) was added for 1 h at 37 °C. After PBST washing, serum (1:400 dilution) was added for 1 h at 37 °C. After plate-washing, HRP-goat anti-mouse IgG (H+L) (1:2 000; Santa) and IgG1, IgG2a (1:1 000; Santa) were added accordingly for 1 h at 37 °C. Absorbance was detected at the absorbance of 450 nm using soluble TMB color solution.

2.9. Cytokine analysis

Spleen was collected from each group 2 weeks after each immunization. Splenocyte suspension was prepared based on the previous descriptions [28] and was spread within 24-well plates, with 5×10^6 cells in 500 μL of culture medium per well. Splenocytes were stimulated for 72 h using recombinant protein (10 μg/mL) and positive control was stimulated using ConA (5 μg/mL). IFN-γ and IL-4 in the cultural supernatant were detected using ELISA kit (450 nm, Thermo).

2.10. Evaluation for erythrocyte-stage preventive effect

Two weeks after the final immunization, abdominal erythrocytes of each mouse were infected by 10^6 *P. berghei* in both groups (ten mice in each group). Tail blood of mice was collected for smearing 1 d after being infected, with giemsa staining. Under themicroscope, malaria was detected until the death or recovery of mice.

2.11. Analysis of ookinete development in vitro

Enterocoelia of mice was infected by 1×10^7 plasmodium after phenylhydrazine processing. Anticoagulation was collected 3 d after being infected and every 20 μL blood was added into 180 μL ookinete culture medium. This culture was added to 96-well plates (contained anti GAMA mice serum and control immune serum) for 24 h at 19 °C, with the serum dilution ratio 1:5, 1:10 and 1:50. After nurturing, this culture was collected centrifugally (500 ×*g*, 5 min) and was washed for once using PBS. After its fixation, Pbs28 McAb 13.1 was used for marking and ookinete numbers were counted under fluorescence microscope.

2.12. Transmission-blocking analysis of direct mosquito bites (Direct feeding assay)

As for 3 mice in each group after immunization, phenylhydrazine processing was carried out 10 d after the final immunization. Three days after that, erythrocytes of mice were infected by 10^6 *P. berghei*. Then 50 *A. stephensi* which had been in hunger for 24 h in each group were used to bite the mice. Mosquitoes were bred using 10% of sugar water under the humidity of 50%–80% and (19–22) °C. Ten days after being infected. Mosquito stomach was dissected and oocyst numbers were counted. Compared with VR1020 control group, percentage loss of oocyst was counted to analyze the blocking levels.

2.13. Analysis of liver-stage worm load in vivo

Caudal vein of mice (3 mice/group) was injected with 1×10^4 sporozoite of *P. berghei*. About 42 h after being infected, mice were anaesthetized using chloral hydrate and were put to death by dislocation of cervical vertebra. The whole liver of mice was obtained and was put into RNAiso Plus (TaKaRa) for grinding and blending. After RNA extracting, inverse transcription (PrimeScript RT reagent Kit, TaKaRa) was carried out. Reagent-SYBR Premix EX *Taq*II (TaKaRa) was used for real-time quantification PCR and analysis. Each sample was repeated for 3 times. Specific primers: *P. berghei* 18S 5′-GGAGATTGGTTTTGACGTTTATGTG-3′; 5′-AAGCATTAAATAAAGCGAATACATCCTTAC-3′ and mice *GAPDH* 5′-CGTCCCGTAGACAAAATGGT-3′; 5′-TTGATGGCAACAATCTCCAC-3′.

2.14. Statistical analysis

GraphPad Prism5.01 software was used for statistical analysis. Mann-Whitney-*U* test or Student's *t*-test were used for

statistical comparison between groups, where $P < 0.05$ indicated the statistical significance.

3. Results

3.1. Bioinformatics analysis

Target protein was obtained by PlasmoDB database screening. Screening conditions: 1. Target protein had membrane related transmission components like signal peptide, transmembrane domain, GPI-anchored and plasmodium; 2. Target protein predicted and involved in the interaction between adhesion & invasion protein and protein. The screening GAMA caused the attention due to its multi-stage expression. GAMA of *P. berghei* consisted of 625 amino acids, with molecular mass 71.1 kDa, signal peptide 1-21 amino acid residue and transmembrane domain: 602-625 amino acid residue. Multiple sequence comparative analysis showed that GAMA was relatively conservative and the consistency of which reached to 57.4% between parasite species. Two highly conservative segments were contained in GAMA. Immunogenicity prediction software indicated that GAMA had better antigenic characteristics.

3.2. Successful building of recombinant DNA vaccine

22-601 amino acid segments of PbGAMA were connected to vector VR1020 and recombinant DNA vaccine VR1020-PbGAMA was built, with correct enzyme digestion identification and sequencing. Western blot analysis showed that antiserum which was generated by DNA vaccine immunity could be combined with *Escherichia coli* expressive recombinant protein (rPbGAMA, 77 kDa), with no stripes in empty vector group. Indirect immunofluorescence also showed that antiserum could be combined with merozoite, ookinete and sporozoite of *P. berghei*. Fluorescence could not be detected in control group. These phenomena and results indicated that VR1020-PbGAMA DNA vaccine could be expressed effectively *in vivo* and could stimulate the body to produce antibody which could recognize the PbGAMA.

3.3. Cloning, expression and purification of PbGAMA protein

Segments with higher antigenicity (46-552 amino acid) were selected based on antigen peptide prediction results. Recombinant protein-PbGAMA was expressed in soluble form and was purified under natural conditions. Protein which was purified by SDS-PAGE electrophoresis was estimated at 77 kDa. Recombinant protein could be recognized by the tag antibody of anti-histidine.

3.4. Antibody responses induced by immunization

The total IgG levels had no obvious changes in PBS group and empty vector group. The total IgG levels in VR1020-PbGAMA immune group significantly rose, compared with two control groups after the second immunization ($P < 0.05$). IgG subclass levels in VR1020-PbGAMA immune group also significantly rose, compared with control group ($P < 0.05$).

3.5. Analysis of cytokine IL-4 and INF-γ induced by immunization

The immune responses of constructed DNA vaccine were analyzed and mice in three groups were immunized with PBS, VR1020 and VR1020-PbGAMA respectively. The IL-4 and INF-γ levels of splenic cell culture supernatant were detected by ELISA kit two weeks after each immunization. IL-4 and INF-γ levels in VR1020-PbGAMA DNA vaccine immunity group significantly rose, compared with control group ($P < 0.05$).

3.6. Evaluation on erythrocyte-stage preventive effect in vivo

Tail blood smearing detection showed that malaria curve was similar to that in control group. One day after being infected, the infected erythrocytes in each group could be found in peripheral blood. Malaria in three groups went up gradually and reached to the highest level at day 10. This highest level was maintained for several days until the death of mice. Survival rate between groups had no difference. Eleven days after being infected, mice began to die gradually; no survival mice existed in each group 21 d after being infected. The results indicated that VR1020-PbGAMA DNA plasmid immunization could not effectively delay or lower the malaria infected by *P. berghei* and extend the survival time.

3.7. Evaluation on the transmission-blocking ability

Ookinete conversion rate was detected *in vitro* to measure the development and viability of ookinete (contained antiserum). Compared with control group, ookinete numbers were not obviously reduced in 24 h ookinete culture with final antiserum concentration 1:5; 1:10 and 1:50. Further transmission-blocking effect was detected to explain whether antiserum could affect the recognition and trajection of ookinete and peritrophic matrix and microvilli. *A. stephensi* in each group were used to bite the mice (infected by *P. berghei* for 3 d) accordingly. The oocyst density of mosquito stomach in PBS and empty vector group were 41.2 and 35.4, respectively. The oocyst density in study group was 40.0, which was not significantly reduced compared with control group.

3.8. Evaluation on the liver-stage protection

P. berghei 18SrRNA could be detected in uninfected control group. Compared with infected control group, liver worm load was slightly reduced in vaccine immunity group. The remaining 5 mice in each group were detected with infected erythrocytes 4 d after being infected. The results showed that VR1020-PbGAMA DNA vaccine immunization could partly block the sporozoite invasion and development, but could not prevent from the blood infection.

4. Discussion

Malaria has brought great threat to the public health and economic development in the worldwide. After more than half a century of centralized researches, only one malaria candidate vaccine ‘RTS,S’ may be registered on the market. However, its

efficacy for preventing the occurrence of clinical cases was limited (46% for children with 18 months and only 27% for infants) [6]. Due to the fact that vaccination without complete immune protection effect could increase the transmission of high virulence pathogens [29], the next generation of malaria vaccine with higher transmission-blocking effect was in urgency. This paper was aimed to illustrate the feasibility of GAMA as multi-stage candidate vaccine and illustrate whether GAMA could produce pre erythrocyte-stage and erythrocyte-stage protective efficacy and had transmission-blocking ability after immunization.

GAMA had features of candidate vaccine. The antigen diversification of plasmodium was one of the main causes to inhibit the malaria vaccine research [30]. Multiple sequence alignment analysis proved that GAMA was relatively conservative between species of plasmodium and had two highly conservative segments. GAMA was synthesized as precursor protein and then was processed for two segments using protease digestion, which was in accordance with reports in literature [22,23]. In addition, bioinformatic prediction indicated that GAMA had better antigenic properties, which could stimulate the body to produce specific immune responses. Recently, individual immune serum of attenuated sporozoite was irradiated by X-ray, GAMA was screened as pre erythrocyte-stage antigen and had value of vaccine research [31].

Anti-malarial protection was related with Th1 and Th2 mediated immune responses. Th1 cell produced the IL-2 and IFN-γ and adjusted the producing of IgG2a; while Th2 cell secreted the IL-4, IL-5, IL-10 and IL-13 and assisted the secretion of antibody IgG1 and IgE [32]. In our study, IFN-γ, IL-4, IgG1 and IgG2a levels were significantly increased, compared with control group. In addition, IFN-γ responses were more obvious than IL-4 responses, with approximately 4 times of increase. These results indicated that PbGAMA immunization could stimulate the body to produce specific immune responses and DNA immunization could produce relatively stronger Th1 assistant immune responses. A few preventive effects which were observed at pre erythrocyte-stage may be related with such immune responses.

In our study, recombinant GAMA DNA vaccine was inoculated, which produced antigen specific immune responses, but could not play better preventive effect on the invasion of *P. berghei* and did not induce the immunity of transmission-blocking. Although mechanism (immunoprotection could not be built) has not be clear yet in this paper, possible reasons were as followed: With reference to anti circumsporozoite protein antibody, when antibody titer was 51 U/mL, it had 50% of efficacy for preventing from children infection and clinical cases [33]. Therefore, a large number of high affinity antibodies were necessary to reach physiological measurable anti-malarial effect. Also, the access of invasion between mediated parasites and host was various and was overlapped functionally [34]. GPI-anchored proteins was overlapped functionally [35,36]. In such circumstances, antibody combined with deciduous GAMA could reduce the antibody mediated cytotoxic effect. What's more, translation codon should be considered. AT content was higher in plasmodium gene group and codon optimization could promote the expression of DNA vaccine *in vivo* and could increase the antigen responses [37]. Besides, we thought that the opportunity of antibody mediated transmission-blocking effect should be very important. Complement and phagocytes were included in vertebrate animal host components which inhibited the zygote survival in blood meal [38,39]. In the internal environment of mosquito stomach, the effects of these factors would be reduced gradually. Therefore, antigen expressed at the early sexual stage may have better transmission-blocking effect. Immunological effect dynamics in the mosquito stomach derived from vertebrate animal hosts has not been reported yet. The opportunity of GAMA expression at sexual stage was also need to be confirmed in the future.

The role and importance of GAMA were different in different plasmodium. The *GAMA* gene of *Plasmodium falciparum* 3D7 and W2mef could not be knocked out, while isogenous *GAMA* gene of *P. berghei* could be knocked out [20–22]. We also found similar phenomena that compared with *P. berghei*, DNA vaccine immunity had more obvious preventive effect on erythrocyte-stage invasion of *P. yoelii*. Under similar antibody responses, the preventive effect on *P. berghei* was more difficult to reach, compared with *P. yoelii* [40]. This difference between *P. berghei* and *P. yoelii* could be used to partly explain why ideal immunoprotection against to *P. berghei* has not been built yet. What's more, some antigens could arouse effective immunoprotection responses alone. However, these antigens combined with other antigens could significantly increase the immunoprotection responses [41]. Anti GAMA antibody combined with anti erythrocyte-binding antigen 175 antibody had obvious high-level transmission-blocking ability for erythrocyte-stage invasion [22] and had a certain protective efficacy at pre-erythrocytic stage. Therefore, further GAMA vaccine research should not be excluded.

In conclusion, the feasibility of GAMA as candidate vaccine also needed a further exploration. Experimental analysis of its applicability and value in multicomponent combined vaccine was necessary. Besides, it was necessary to change the immunoassay platforms to guide other effect mechanisms.

Conflict of interest statement

We declare that we have no conflict of interest.

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